N-Nitroso Compounds

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ACS Symposium Series 174

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Based on a symposium
cosponsored by the Divisions of
Agricultural and Food Chemistry
and Pesticide Chemistry
at the 181st Meeting of the
American Chemical Society,
Atlanta, Georgia,
March 31–April 1, 1981.

ACS SYMPOSIUM SERIES 174

AMERICAN CHEMICAL SOCIETY
WASHINGTON, D. C.

Library of Congress CIP Data

N-nitroso compounds.
(ACS symposium series, ISSN 0097–6156; 174)

"Based on a symposium cosponsored by the Divisions of Agricultural and Food Chemistry and Pesticide Chemistry at the 181st meeting of the American Chemical Society, Atlanta, Georgia, March 31–April 1, 1981."

Includes bibliographies and index.

1. Nitroso-compounds—Congresses. 2. Nitrosoamines—Congresses. 3. Carcinogens—Congresses. 4. Nitroso-compounds—Metabolism—Congresses.
I. Scanlan, Richard A., 1937– . II. Tannenbaum, Steven R., 1937– . III. American Chemical Society. Division of Agricultural and Food Chemistry. IV. American Chemical Society. Division of Pesticide Chemistry. V. Series.

RC268.7.N58N18 616.99'4071 81–19047
ISBN 0–8412–0667–8 AACR2
ACSMC8 174 1–400 1981

PRINTED IN THE UNITED STATES OF AMERICA

ACS Symposium Series

M. Joan Comstock, *Series Editor*

FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable since symposia may embrace both types of presentation.

PREFACE

It is approximately twenty-five years since John Barnes and Peter Magee discovered that dimethylnitrosamine was a carcinogen for the rat. Since that time *N*-nitroso compounds have come to be among the most important experimental mutagens and carcinogens for laboratory investigation. Equally, if not more importantly, they have come to be viewed as one of the most important classes of environmental carcinogens, and the potential for endogenous formation from ubiquitous precursors has been recognized. Their presence and the presence of their precursors in foods, water, air, and industrial and agricultural products has led to frantic calls for legislative and regulatory action, and a never-ending search for more sensitive and specific methods of analysis.

The symposium upon which this volume is based was organized at a turning point in nitrosamine research. Almost all types of commercial products have been tested for volatile nitrosamines, and there have been a number of outstanding accomplishments of combined university–government–private industry actions to lower or eliminate volatile nitrosamines in those products found to be contaminated. However, there is still a major gap of knowledge with regard to compounds that are not amenable to analysis by gas chromatography, and this is clearly a frontier of current research. There are also many important questions regarding chemistry, mechanism of action, and relation to human disease whose answers lie in the future of research in this field.

It is the purpose of this volume to summarize the current state of knowledge of nitrosamine research with a chemical orientation, and to help lead the way into the future.

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July 31, 1981

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CHEMISTRY AND METABOLISM

Activation of Nitrosamines to Biological Alkylating Agents

C. J. MICHEJDA, M. B. KROEGER-KOEPKE, S. R. KOEPKE, and D. H. SIEH

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N-Nitrosamines require metabolic activation to generate the reactive species which result in tumor induction. The most commonly accepted hypothesis for this activation is the α-hydroxylation hypothesis. Nitrosamines which have hydrogens on the alpha carbons are hydroxylated in that position by a mixed function oxygenase. The resultant α-hydroxyalkyl nitrosamine breaks down to an alkyldiazonium ion and the corresponding carbonyl compound. The diazonium ion alkylates a variety of nucleophiles and releases molecular nitrogen. The determination of the amount of molecular nitrogen provides a measure of the extent of α-hydroxylation. This concept was applied to the *in vitro* metabolism of doubly [^{15}N]-labeled dimethylnitrosamine (DMN) and N-nitrosomethylaniline (NMA) by uninduced rat liver S-9 (the postmitochondrial fraction). The amount of total metabolism was determined by the measurement of nitrosamine loss. It was found that measurement of formaldehyde formation gave an artificially low value of nitrosamine metabolism. The *in vitro* results indicated that about 34% of the DMN metabolism proceeded by a α-hydroxylation, while only 19% of the theoretical nitrogen was released by NMA. A similar experiment *in vivo*, using [^{15}N]-labeled DMN showed that about 66% of the metabolism proceeded by α-hydroxylation.

Alternative pathways of activation of nitrosamines, including β-hydroxylation followed by sulfate conjugation and the formation of alkoxydiazenium ions are discussed. The formation of alkyldiazonium ions from trialkyltriazenes is presented to show that the formation of the putative ultimate carcinogens from nitrosamines can be studied in a system not requiring metabolic activation.

0097-6156/81/0174-0003$05.00/0

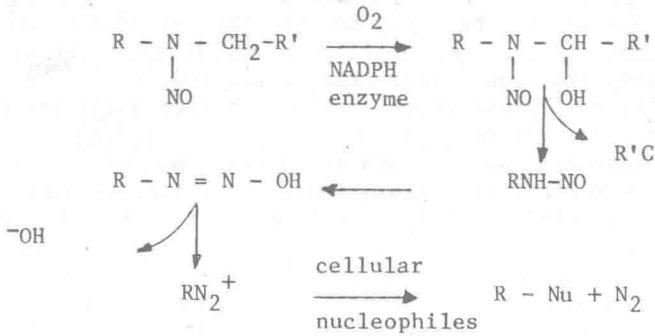
In 1956 Magee and Barnes (1) reported that dimethylnitrosamine (DMN) was a potent carcinogen in rats. This discovery initiated a mighty outburst of research activity. There are now thousands of papers dealing with the chemistry, metabolism, mutagenicity, teratogenicity, and carcinogenicity of nitrosamines and other N-nitroso compounds. There are several reasons for this continuing interest. Practically all nitrosamines are carcinogenic, making them the largest single chemical group which has that property. In fact, non-carcinogens among them may provide important clues to what makes nitrosamines carcinogenic. Nitrosamines are remarkably site specific; essentially all the organs are effected by one or another nitrosamine (2). This makes nitrosamines excellent tools for the study of mechanisms of chemical carcinogenesis. Perhaps most importantly, nitrosamines can be and are formed in the human environment. The reason for this is that the two precursors to nitrosamines, nitrite and amines, are ubiquitous components of the environmental mix. Moreover, nitrosamines can be formed in the stomach by ingesting the precursor amines and nitrite. Several chapters in this volume concern themselves with environmental and dietary aspects of nitrosamines.

The first ten years of nitrosamine research, particularly with respect to carcinogenesis, were summarized in an admirable review by Magee and Barnes (3). Since that time an enormous amount of work has been carried out on those substances. Some of this work up to 1974 was reviewed by Magee, Montesano and Preussmann (4). Recently, Lai and Arcos (5) provided a useful synopsis of contemporary work on the bioactivation of some selected dialkylnitrosamines.

Nitrosamines, in common with many other organic carcinogens, have to be metabolized before their carcinogenic potential can be expressed. Thus, Magee (6) in 1956 showed that dimethylnitrosamine (DMN) was cleared rapidly from the bodies of rats, mice, and rabbits, with very little being excreted in the urine or the feces. Dutton and Heath (7) reported that [^{14}C]-DMN was metabolized in rats and mice, with a major portion of ^{14}C appearing in expired carbon dioxide, with the rest of the radioactivity being evenly distributed in the tissues. Only about 7% of the radioactivity was found in the urine. These workers also postulated that the metabolism proceeded by a demethylation reaction and concluded that the lesions produced by DMN were the result of the interaction of metabolites of DMN with cellular components, rather than with the intact DMN. Since the liver was the principal target of DMN, Magee and Vandekar (8) used liver slices and subcellular fractions to study the *in vitro* metabolism of DMN. They found that the metabolizing activity was localized in the microsomes and in the cytosol and that it required molecular oxygen and the presence of NADPH. The formation of formaldehyde from DMN was demonstrated by Brouwers and Emmelot (9). The principal enzyme system responsible for

DMN oxidative demethylation has been shown to be a liver microsome cytochrome P-450 monooxygenase (10). Lotlikar et al. (11) found that a reconstituted enzyme system, consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase and phosphatidyl choline was effective in catalyzing the demethylation of DMN. The most commonly accepted mechanism for the oxidative demethylation of DMN and, by extension, of other dialkylnitrosamines is shown in Scheme 1.

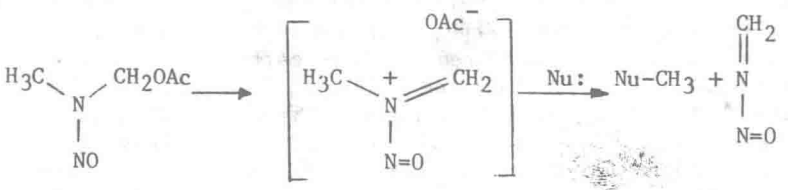
Scheme 1



The α-Hydroxylation Hypothesis

The above scheme satisfies much of the metabolic data; however, some of it is speculative, and it is certainly incomplete. The evidence for the formation of the α-hydroxylated intermediate is circumstantial. The acetate ester of α-hydroxylated dimethylnitrosamine has been prepared (12,13) and has been found to be a potent, directly acting carcinogen (14). Other esters of a variety of α-hydroxylated nitrosamines have also been prepared (15). While it has been shown that DMN acetate is hydrolyzed to hydroxymethylmethylnitrosamine by an esterase enzyme, it has been pointed out that these derivatives of the α-hydroxylated nitrosamines also dissociate to N-nitrosoimmonium ions (15, 16).

Wiessler (15) suggested that the nitrosoimmonium ion could act as the electrophile which alkylates cellular nucleophiles.



Gold and Linder (17) studied the esterase catalyzed hydrolysis of ℓ-(-)-acetoxymethyl-(1-phenylethyl)nitrosamine. They found that the stereochemistry of 1-phenylethanol produced in the reaction was the same as that observed in the base catalyzed hydrolysis of the nitrosamine and also of N-(1-phenylethyl)nitrosocarbamate. These results indicated that the same diazotate was produced in all three reactions. The fact that no irreversible inhibition of the enzymatic hydrolysis of the nitrosamine was observed, while extensive irreversible inhibition was obtained with the nitrosocarbamate, led these workers to conclude that the α-hydroxynitrosamine produced by the hydrolysis had sufficient stability to diffuse away from the active site of the enzyme.

Recently, the preparation of the first authentic α-hydroxylated nitrosamines has been reported (18,19). N-Butyl-N-hydroperoxymethylnitrosamine (20,21) was reduced with with sodium bisulfite or deoxygenated with triphenylphosphine. The resulting, rather unstable product, was converted to the α-acetoxy derivative with acetic anhydride and it was shown to form formaldehyde and 1- and 2-butanol when allowed to decompose in an aqueous medium.

All of these results support the α-hydroxylation hypothesis, but they do not prove it. No α-hydroxy nitrosamine or a derivative of one has ever been isolated as a metabolite of any nitrosamine. Nevertheless, α-hydroxylation is an attractive working hypothesis and it seems to account for many observed facts, at least in the case of the simplest nitrosamines. One of the salient features of Scheme 1 is that the α-hydroxylated nitrosamines decompose non-enzymatically to the unstable monoalkylnitrosamine, which then undergoes a prototropic shift to the corresponding diazotic acid. This substance (or its dissociation product, the alkyldiazonium ion) is the electrophile that alkylates cellular nucleophiles. Many authors write the electrophile as the corresponding carbenium ion. In most cases, this is incorrect; carbenium ions such as methyl or ethyl are high energy molecules and it is highly unlikely that they are formed in an aqueous medium. Exceptions may exist for such stabilized ions as benzyl or t-butyl, but not for primary, unstabilized carbenium ions. In fact, it was shown that the alkylation of hepatic DNA in rats by di-n-propylnitrosamine gave exclusively 7-n-propylguanine, rather than 7-isopropylguanine, which would have been predicted if free carbenium ions were involved (22). The RNA from these livers, however, did show that about 5% of the 7-propylguanine was the isopropyl isomer.

Whatever the true details of the metabolic pathway shown in Scheme 1 might be, there are certain facts which are very secure. Among these are that the nitrosamines are oxidatively dealkylated, that electrophilic intermediates which alkylate proteins and nucleic acids are formed, and that one of the

products of the dealkylation is a carbonyl compound, formaldehyde in the case of DMN. One consequence of Scheme 1 is that α-hydroxylation obligatorily results in the formation of molecular nitrogen. The measurement of the released nitrogen, in principle, allows an accurate determination of the extent of α-hydroxylation. Magee (23) was the first to indicate the possibility of use of [^{15}N]-labeled nitrosamines in the measurement of the extent of α-hydroxylation. In this work, Holsman, Haliday and Magee measured the formation in rats of ^{15}N-^{15}N gas. The animals were dosed with doubly [^{15}N]-labeled dimethylnitrosamine, while being maintained in a respiration chamber whose atmosphere was 50% oxygen and 50% sulfur hexafluoride. After 5 hrs, the rats were killed and the expired gas was put through a set of scrubbers and cold traps to remove O_2, CO_2, SF_6, and other condensibles. The residual gas, consisting largely of nitrogen, was analyzed by mass spectrometry. They found that a major portion of the DMN was metabolized by the α-hydroxylation pathway, since up to 90% of the total applied dose was found in the expired nitrogen. These results, however, were considered to be preliminary.

Cottrell et al (24) studied the in vitro metabolism of [^{15}N]-labeled dimethylnitrosamine. They found that the 10,000g supernatant fraction (usually called the S-10 fraction) of rat liver homogenates produced labeled nitrogen at the rate of 5% of the total metabolism of the nitrosamine as measured by the production of formaldehyde and methanol. These workers concluded that α-hydroxylation was relatively unimportant during in vitro metabolism. In stark contrast to these results, Milstein and Guttenplan (25), using unlabeled dimethylnitrosamine, found that essentially 100% of the microsome-catalyzed metabolism of DMN proceeded by α-hydroxylation. Against the backdrop of these seemingly contradictory results, we have undertaken a systematic examination of both the in vivo and in vitro metabolism of [^{15}N]-labeled nitrosamines. These experiments are being carried out in collaboration with Dr. P.N. Magee of the Fels Institute. In the initial experiments we chose to concentrate on doubly [^{15}N]-labeled dimethylnitrosamine and N-nitroso-N-methylaniline (NMA).

The in vitro experiments, using the S-9 fraction from livers of uninduced Fisher 344 rats, was complicated by the fact that it became apparent that formaldehyde production was a poor measure of the extent of metabolism. The reason for that was that the S-9 fraction apparently catalyzed the oxidation of formaldehyde to formate. Consequently, determination of formaldehyde in an S-9 catalyzed reaction consistently gave low values of nitrosamine metabolism. Many workers use semicarbazide to suppress formaldehyde loss. We found, however, that semicarbazide is not a neutral bystander,

because it itself acts as a substrate for the S-9 enzymes, giving molecular nitrogen as a product. The difficulties with semicarbazide as a formaldehyde protector in S-9 catalyzed reactions were also independently noted by Saveni je-Chapel and Noordhoek (26). Since formaldehyde formation was inadequate as an indicator of metabolism, we chose to follow the metabolism by determining substrate loss. This is an inherently imprecise measurement because the amount of total metabolism is small. Nevertheless, we found that with appropriate sample pre-treatment, reasonably reproducible data could be obtained. The S-9 catalyzed reactions were carried out in calibrated flasks. The reaction atmosphere was oxygen containing a precisely known amount of neon. After one hour reaction, the gas was transferred by means of a Toepler pump into a bulb, the contents of which were analyzed on a mass spectrometer. The instrument was calibrated against precisely known mixtures of ^{15}N-^{15}N and neon in oxygen. By knowing the total amount of gas in the reaction mixture, the absolute amount of ^{15}N-^{15}N evolved during the reaction could be calculated. The reaction mixture itself was assayed at the start of the reaction and after one hour by high pressure liquid chromatography (hplc). In order to get reproducible results in the hplc experiment it was necessary to filter the reaction mixture through a membrane ultrafilter, which excluded the high molecular weight materials. The results of these experiments on doubly [^{15}N]-labeled DMN and NMA are presented in Table I.

Table I.

Analysis of Metabolism of [^{15}N]-Labeled DMN and NMA by the S-9 Fraction[a]

Substrate	Substrate Lost (μ moles/g liver/hr)	HCOH Detected (μ moles/g liver/hr)	^{15}N-^{15}N-Produced (μ moles/g liver/hr)
DMN	0.732 ± .184(5)	0.169 ± .050(6)	0.240 ± .031(4)
NMA	1.570 ± .173(5)	0.039 ± .010(4)	0.296 ± .074(3)

[a]Results expressed as the average ± standard deviation (number of observations)

These results indicate that in the in vitro reaction, the α-hydroxylation pathway accounts for about 34% of the metabolism of DMN and about 19% of the metabolism of NMA, when uninduced Fisher 344 rat liver S-9 is used. Thus, our data for DMN fall roughly in-between the two previously published

values (24,25). The data also clearly indicate that the formaldehyde assay gives erroneous results for the total amount of metabolism, when the S-9 fraction is used as the oxidation catalyst.

The present data give us no indication of what the other pathways of DMN metabolism might be. Other pathways, including denitrosation and reduction of the NO group to the unsymmetrical hydrazine have been reported (27).

The *in vivo* metabolism of [^{15}N]-labeled DMN was carried out by a method similar to the one developed by Holsman, Haliday and Magee (23). A rat was injected IP with 276 μ mole/kg of doubly [^{15}N]-labeled DMN and was placed in a respiration chamber equipped with a collapsible top. The chamber was charged with an atmosphere of SF_6, oxygen and a known amount of neon as an internal standard. The entire system was calibrated with precisely known mixtures of ^{15}N-^{15}N, neon, and oxygen. After 6 hrs, the rat was killed by an injection of chloroform into the chamber and the gas in the chamber was pumped through a series of traps to remove the condensible gases. The residual gas was analyzed by mass spectrometry. This *in vivo* experiment was carried out at the Fels Institute by Ms. Cecilia Chu and Dr. Magee. The gas analysis was carried out at Frederick. The results are presented in Table II.

Table II.

Analysis of the *in vivo* Metabolism of [^{15}N]-Labeled DMN

moles[a] of ^{15}N-DMN injected per rat x 10^5	moles of ^{15}N-^{15}N evolved[b] x 10^5	% of metabolism resulting in ^{15}N-^{15}N
3.04	1.81	60
4.03	3.18	79
4.03	2.08	52
4.14	3.08	74
	Average ± SD =	66 ± 12

[a]Each animal received an equimolar dose of 276 μmole/kg. This dose was determined by Magee to be metabolized completely within 6 hrs.

[b]The determination was made using neon as an internal standard.

It is clear from the results in Table II that α-hydroxylation accounts for a major part of the metabolism of DMN *in vivo*. The percentage is a little lower than the preliminary values obtained by Halsman, Haliday and Magee (23), but it is